Tumor control and normal tissue toxicity: The two faces of radiotherapy
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Citation for published version (APA):
van Oorschot, B. (2016). Tumor control and normal tissue toxicity: The two faces of radiotherapy
CHAPTER 1

General Introduction

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*DNA Repair Genes and Late Toxicity after Radiotherapy,*  
*Turkiye Klinikleri - Radiation Oncology-Special Topics, 2015; 1(3):8-15*
RADIOTHERAPY

Curative cancer treatment aims to efficiently eliminate cancer cells, leading to the control of the tumor and eventually to the cure of the patient. Besides surgery and chemotherapy, radiotherapy is one of the three main methods for the treatment of cancer. Radiotherapy uses ionizing radiation to induce lethal DNA damage in locally targeted cancer cells, while sparing normal, healthy cells as much as possible [1]. Unfortunately, radiotherapy is, like most cancer treatments, not specific for tumor cells and can also induce damage to normal tissues. Although rapidly dividing cancer cells are thought to be more sensitive to radiation then normal cells, the desired radiotherapy dose is limited by the maximum tolerance of the surrounding normal tissue [2]. Over the years, technical innovations have significantly improved the practice of radiotherapy. Modern anatomic imaging technologies, e.g. computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET), have been a major impetus to the ongoing development of conformal radiotherapy techniques. High precision radiotherapy allows for a better identification of tumor volumes and their spatial relationship with vital organs [3, 4]. Subsequently, tumors can be targeted with higher radiation doses leading to improved tumor control and the radiation dose in surrounding tissues remains minimized, thereby reducing the risk of side effects [5]. Furthermore, radiation treatment is applied in multiple fractions over time. Fractionation of the total radiation dose, gives normal cells time to recover and repair their DNA damage whilst cancer cells are still destroyed efficiently. As cancer cells are thought to repair their DNA damage less accurately [6], the time between radiation fractions opens a therapeutic window for dose escalation and efficient tumor kill. This biological advantage of healthy cells over cancer cells largely explains the effectiveness of cancer radiotherapy.

Despite advances in tumor imaging, dose-escalations and targeting, many tumors are poorly controlled by radiotherapy alone. Therefore, radiotherapy is frequently combined with other treatment modalities [7] to enhance the effect of radiation treatment [1]. The technological advances together with a better understanding of various molecular mechanisms involved in the radiation response of the tumor [8, 9], have led to increased local tumor control rates and a better overall survival of cancer patients [10]. The increase in the number of cancer survivors and life expectancy however, also raised the importance of quality of life after treatment. This means that not only improvements in tumor control are warranted, but that also the prevention or prediction of severe side effects becomes a necessity. Moreover, there are significant variations between individuals in their response to therapy, both in tumor and normal tissues. This asks for the need of personalized treatment strategies: screening in advance which treatment might be most beneficial for the individual patient. As radiotherapy is used in approximately 50% of all cancer treatments [11], approaches that can improve tumor control or
decrease normal tissue toxicity will affect many cancer patients. Prediction of tumor and normal cell sensitivity to ionizing radiation can be used to further optimize and individualize treatment strategies.

This thesis will discuss the molecular responses to ionizing radiation in tumors and in normal tissues. The DNA damage response is highlighted as the main mechanism that can influence the efficacy and outcome of radiation treatment. Part I (chapters 2, 3, and 4) will discuss the radiation response in several cancer cell types and how the DNA damage response can be used to interfere with cellular sensitivity to radiation treatment. Part II (chapters 5 and 6) will address the development of normal tissue toxicity after radiation treatment. Here, the DNA damage response in normal cells is elaborated as possible predictor of late radiation toxicity in prostate cancer patients. This introductory chapter 1 will provide a general overview of the biological responses in mammalian cells after exposure to ionizing radiation and outline the specific challenges in studying the radiation response in cancer cells and in normal cells.

DNA DAMAGE RESPONSE

Damage to a cell’s DNA is the main cause of cell death induced by ionizing radiation. Ionizing radiation can damage the DNA by direct deposition of high energy on the DNA and indirectly by ionization of water molecules to produce hydroxyl radicals that attack the DNA [12]. Exposure to ionizing radiation may cause several types of DNA-damage: single strand breaks (SSBs), double strand breaks (DSBs), base damage and DNA-protein cross-links in the genomic DNA. Among those, DSBs are the most deleterious type of DNA damage [13] and the yield of radiation-induced DSBs increases linearly with the radiation dose [14, 15]. Usually, the dose per fractions applied during radiation treatment ranges between 1.5-2.5Gy, and depending on cell type 20 to 40 DSBs/cell per Gray are induced [16].

As DNA lesions occur frequently in mammalian cells due to a variety of endogenous or exogenous processes [17, 18], cells have evolved a multifaceted response to DNA damage. The DNA damage response includes cell cycle checkpoint activation to arrest cells in replication or division and the induction of DNA repair mechanisms (Figure 1). When the damage is too severe for the repair mechanism to cope with, damaged cells might go into senescence, undergo mitotic cell death or initiate apoptosis [19]. These natural responses to DNA damage aim at preserving genomic integrity and hence cell viability. As previously stated, DNA DSBs are the most lethal kind of DNA damage induced by radiation, and failure to repair even a single DNA DSB could result in genomic instability, mutagenesis, aging or cell death [20, 21]. Moreover, unrepaired DSBs can contribute to various disorders, including carcinogenesis [17, 22].
Figure 1. Schematic overview of the DNA damage response after radiation exposure. After the induction of DNA double strand breaks, cell proliferation is halted, severity of the damage is assessed and if possible repaired. Misjoining of DNA breaks might result in chromosome translocations, mutations or cancer, whereas correct repair leads to cell survival. Unrepaired breaks will eventually lead to senescence or cell death.

For proper functioning and survival of a cell, DSBs must therefore be quickly detected and repaired. In normal, healthy cells this process is required; however repair of DSBs in tumor cells could interfere with treatments that aim to destroy cancer cells by inducing lethal DNA damage. Consequently, the efficacy of the DNA damage response plays an important role in determining tumor sensitivity and outcome of radiation treatment.

Detection and repair of DSBs
At a molecular level, the repair of DSBs is the result of an intricate interplay of specific repair proteins. A critical cellular response to DNA damage, is the activation of ataxia-telangiectasia-mutated (ATM) by the Mre11-Rad50-Nbs1 (MRN) complex [23]. ATM is a serine-threonine kinase protein and interacts with several proteins, including DNA-dependent protein kinase (DNA-PK), AT and Rad3-related (ATR) and Nbs1 [24]. ATM phosphorylates various key regulators of the DNA damage response, like p53, H2AX, BRCA1, and Nbs1 [25]. Tumor suppressor p53 is one of the key players in the activation of G1/S and G2/M cell-cycle checkpoints after DNA damage [26, 27], and triggers various cellular responses like cell cycle arrest, differentiation, DNA repair (Chapter 2) [28] and apoptosis [29]. After initial recognition of a DNA DSB and the activation of the cell cycle checkpoints, the DSB repair cascade is then promoted by the phosphorylation of histone variant H2AX by ATM, ATR or DNA-PK in a chromatin region of several megabases around the DSB. Multiple phosphorylated H2AX proteins (γ-H2AX) form a platform, which subsequently attracts other DSB repair proteins to the site of the break [30, 31]. Among others, MDC1, 53BP1 and MRN accumulate closely to the γ-H2AX proteins within minutes after...
induction of a DSB, together forming a so called ionizing radiation induced foci (IRIF) \[32\]. γ-H2AX is, as one of the earliest marker of the DSB repair, essential for efficient repair of DSBs \[33-35\]. Furthermore, γ-H2AX can easily be detected by an immunofluorescence staining making it an important biomarker for DNA DSBs \[32\] (Chapter 4). As defects in IRIF formation are linked to repair deficiencies \[36\], the detection and scoring of these foci has become a valuable tool for many clinical investigations of mutations in DNA repair and the sensitivity of treatments \[37-39\].

Following recognition of a DNA DSB, there are two major pathways for the repair of ionizing radiation induced DSBs, that differ in their requirements for DNA homology: homologous recombination (HR) and non-homologous end joining (NHEJ) \[40-42\]. HR is an accurate ‘error-free’ form of repair, which requires an intact sister chromatid to act as a DNA template and can therefore only function during S- and G2-phase of the cell cycle. In contrast, NHEJ is active throughout the whole cell cycle and is considered to repair the majority of the DSBs in human cells \[43\]. In its simplest sense, NHEJ entails direct rejoining of the DNA ends. Blunt end joining can lead to loss of nucleotides from either side of the break, making NHEJ potentially error prone. Both pathways will be briefly described, as well as the recently described alternative error-prone form of DSB repair (alternative end joining, alt-EJ).

**Homologous Recombination.**

The HR pathway represents the error-free form of DSB repair. Unfortunately, its activity is limited to the S- and G2-phase of the cell cycle due to the requirement of a homologous DNA template. Therefore, HR only accounts for approximately 20% of the DSB repair induced by ionizing radiation \[40, 44\]. HR is triggered by the resection of 5’ DNA end by the MRN complex. After resection, replication protein A (RPA) binds to the 3’ single strand DNA tail (ssDNA) and protects the ends from degradation. As soon as BRCA2 protein binds RAD51, the BRCA2-RAD51 complex replaces RPA on the ssDNA \[45\]. On the ssDNA tail, RAD51 forms long filaments that can bind to the homologous DNA template. During this so-called synapsis phase, promoted by the protein RAD54, a D-loop is created between the resected ssDNA and the DNA template. Finally, the ssDNA tail primes DNA synthesis by DNA polymerase and the break will be repaired.

**Non-Homologous End Joining**

Most of the radiation induced DSBs are repaired via the mutagenic non-homologous end-joining (NHEJ) pathway \[43, 46\]. The NHEJ pathway ligates the DSB ends through a process that is independent of DNA sequence homology, and therefore frequently leads to sequence errors. Although NHEJ is error prone, it most rapidly repairs DSBs and thereby protects the genome integrity. NHEJ pathway is mainly regulated by DNA-PK catalytic subunit (DNA-PKcs), the KU70/KU80 complex, DNA ligase IV/XRCC4 and XLF (cernunnos) \[41, 47\]. The first step in NHEJ repair is the accumulation of the KU70/KU80 heterodimer at the site of a DNA DSBs \[48\]. The
heterodimer encircles the DNA ends, keeping them together for the duration of the break repair, and attracts DNA-PKcs. Thereafter, DNA-PKcs undergoes auto-phosphorylation and activates the recruitment of other NHEJ repair proteins. The second step of NHEJ involves the enzymatic processing of the DSB, that often contain non-cohesive or non-ligatable DNA ends [49]. DNA ends carrying for instance 3' phosphates or 5' hydroxyl groups are polished by the Artemis nuclease and other DNA polynucleotide kinases. Finally, the DNA ends are ligated by the DNA ligase IV-XRCC4 complex together with the XRCC4-like factor (XLF) [50]. The latter has been described to be redundant and not essential for the NHEJ repair [51].

**Alternative End Joining**

In addition to HR and NHEJ, there is increasing evidence for the existence of one or more error-prone alternative end-joining pathways that ligate DNA ends [52]. The detection of translocations in NHEJ deficient cells hinted the activity of some other form of mutagenic repair [52]. It was initially thought that alt-EJ mainly served as a backup repair pathway for NHEJ, as it occurs more slowly, less efficient and leads to more chromosomal translocations than classical NHEJ [53]. However, recent studies suggest that alt-EJ might also compete with HR repair, as both HR and alt-EJ require an initial end resection step and alt-EJ uses small homologous DNA sequences (microhomologies) to repair the break [54]. Small microhomologies can lead to deletions and/or insertions in the DNA, as they not necessarily contain the correct DNA sequence. So far, PARP1, XRCC1, LIG1 and LIG3 have been implicated to be involved in the alt-EJ pathway [55], but a lot is still unknown. Whether alt-EJ processes are only active in the absence of NHEJ and HR or also compete with functional HR or NHEJ repair is not clear and the overall significance of these alt-EJ pathways need yet to be determined [50, 52].
INTRODUCTION TO PART I: RADIATION RESPONSE IN CANCER CELLS

Approaches that aim to improve tumor control by radiation treatment include developments in tumor imaging, defining the different molecular features of tumors and cancer cells to predict treatment response and the identification of specific molecular targets for the development of new drugs [56]. This section will briefly outline the importance of the latter two as they are relevant for the content of this thesis.

As mentioned before, ionizing radiation produces several molecular events in the irradiated cells which can lead to cellular death, a fatal risk that increases with the radiation dose [10]. The probability to induce lethal DNA damage is highest in the M-phase of the cell cycle [57], making rapidly proliferating cancer cells more vulnerable for ionizing radiation compared to normal cells. Moreover, the DNA damage response is thought to be less efficient in cancer cells due to mutations in cell cycle checkpoints and DNA repair genes [1, 7]. Depending on the type of mutation, cancer cells might therefore choose a different repair pathway or rely more on back-up repair processes compared to the repair proficient normal cells. Although an active mutated DNA damage response is also associated with the development of cancer [22], the cancer-specific mutations provide opportunities to selectively target cancer cells. However, heterogeneity within and between tumors poses a challenge for personalized treatment planning, and could hinder correct diagnostics and therapeutic efficacy [58-60]. Furthermore, there are parts of the tumor that are more difficult to treat and/or regions wherein the tumor is less vulnerable to ionizing radiation. In addition, some cancer cells are able to evade or even resist radiation treatment [61]. These surviving cancer cells might cause repopulation of the initial tumor once the cancer treatment stops.

Taken together, the outcome of radiation treatment not only depends on the tumor type and location but also on its molecular phenotype [60]. Insights in the different molecular phenotypes and their specific responses to ionizing radiation opened a window for the development of new targeted drugs. Strategies to combine radiation treatment with molecular-targeted therapies, alongside chemotherapy or surgery, are evolving more and more, enhancing the effect of radiation and increasing tumor control and cure rate.

Resistance to radiation treatment

A common feature in solid tumors is the presence of oxygen-deficient hypoxia regions. Tumor hypoxia negatively influences chemo- and radiotherapy and the level of hypoxia is correlated with treatment resistance and poor survival outcome [62, 63]. Due to the low oxygen levels and reduced perfusion, delivery of chemotherapy or other reagents in this part of the tumor might be minimized. Moreover, hypoxia reduces the indirect effect of ionizing radiation as the production of free radicals and reactive oxygen species (ROS) is limited. This prevents to some
extent the induction of lethal DNA damage [64]. In the presence of oxygen, DNA lesions produced by free radicals can be chemically ‘fixed’, resulting in an irreparable form of DNA breaks. In the absence of oxygen however, this reaction cannot take place and as a results all DNA breaks can be repaired. In addition, an hypoxic environment can also influences the choice of DNA repair processes after initial DSB induction, as it is thought to downregulate the expression of proteins involved in HR repair [65]. This might result in enhanced error-prone repair processes, introducing a more mutagenic or resistant phenotype of the surviving cancer cells [66]. Targeting proteins and genes involved in tumor hypoxia is therefore the focus of many studies and could influence the result of cancer treatment tremendously [67]. On the other hand, treatment strategies that can induce normal tissue processes like angiogenesis are also of interest as they can lead to enhanced tumor responsiveness [68].

Furthermore, hypoxic areas may represent niches for cells possessing stem cell like characteristics [69-71], such as self-renewal, differentiation potential and resistance to apoptotic stimuli. These so called cancer stem cells (CSCs) are involved in tumor initiation and tumor growth and are assumed to be the major cause of recurrence in cancer patients. It is thought that even a single surviving CSC is able to repopulate the initial tumor, highlighting the importance of CSC targeting for treatment outcome. Hypoxia can prevent differentiation of CSCs, thereby promoting the maintenance of the radio-resistant population [71]. Several suggested mechanisms causing treatment-resistance of CSC include an elevated apoptosis threshold, drug-efflux pumps and the induction of quiescence [72]. Furthermore, CSCs are shown to have a high activity of DNA repair pathways [73, 74]. Therefore, a suggested mechanism to sensitize both cancer cells and cancer stem cells to radiation is the inhibition of specific DNA DSB repair proteins (chapter 3).

**Targeting DNA damage response**

The ability of cancer cells to repair lethal DNA damage strongly determines the success of radiation treatment. Targeting proteins of the DNA damage response, especially those of DSB repair, with specific inhibitors is therefore an attractive strategy to eliminate cancer cells [75, 76]. Among others, inhibitors of ATM, PARP1, checkpoint kinases (Chk1/2), and DNA-PKcs have been developed and show promising results in facilitating enhanced cancer cell death after treatment [75, 77], in both *in vitro* cell lines and tumor models *in vivo*.

Molecular inhibitors could affect tumor cells more effectively than normal cells as they can take advantage of the abnormal activated or mutated DNA damage response in cancer cells [22]. For example, PARP1 inhibitors lead to specific tumor kill in BRCA1- and BRCA2 deficient tumors by inducing so-called *synthetic lethality*, i.e. that either PARP1 and BRCA1 deficiency alone is compatible with viability, but together they will lead to cell death [78]. Synthetic lethal approaches could provide treatment strategies that can specifically target tumor cells with
Figure 2. Detection and repair of DNA DSBs induced by ionizing radiation. Proteins involved in the DNA DSB cascade can be used as targets to improve tumor control and treatment efficacy. The targets depicted here are currently being investigated or already implemented in the clinic.

known mutations. Another way to interfere with the efficacy of the DNA damage response is by the use of hyperthermia [79]. Hyperthermia affects the DSB repair HR pathway by temporal degradation of the BRCA2 protein, preventing RAD51 to bind at the DSB ends [80, 81]. In addition, human papillomavirus (HPV) induced cancer types are specifically sensitive for hyperthermia, as the HPV produced oncogene E6 is heat labile [82]. HPV proteins E6 interacts with p53 and perturbs its tumor suppressing functions, leading to the induction of tumor cell growth. Elimination of E6 by hyperthermia enables p53 dependent apoptosis and cell cycle control in these cancer types, re-sensitizing them to chemo- or radiotherapy. Hyperthermia might also affect hypoxic regions by increasing vasodilation and hence oxygen levels, resulting in increasing numbers of DNA damage after radiation. Furthermore, it is an interesting additive treatment option with regard to normal tissue complications, as the addition of hyperthermia to radiation treatment enables lowering of total dose with similar treatment efficiency [83, 84].

In conclusion, the identification of resistant cancer cells and hypoxic tumor areas contribute to a better understanding of treatment response and tumor sensitivity. Characterization of these cell phenotypes can lead to the development of biomarkers and specific molecular-targets for treatment. Furthermore, modification of the DNA damage response and targeting of DSB repair genes may result in enhanced chemo- and radio-sensitization of cancer cells and CSCs. Together they could improve the tumor control significantly and contribute to the development of patient-tailored treatment strategies.
INTRODUCTION TO PART II: RADIATION RESPONSE IN NORMAL TISSUES

Late radiation toxicity of the healthy tissue around the tumor is the limiting factor for dose escalation in radiation treatment. In clinical practice, standardized radiation schedules are applied based on the sensitivity of the average patient. This does not take into account the presence of individuals with increased or decreased sensitivity to radiation. Some patients display severe side-effects despite highly conformal radiotherapy, while others have no problems despite extended radiation fields. Development of methods to predict normal tissue response to radiation treatment therefore represents an important approach to enable individualized treatment planning. For instance, patients at high risk for radiation toxicity may profit from a reduced radiation dose combined with chemotherapy or hyperthermia. Patients at low risk for radiation toxicity may profit from a higher radiation dose leading to higher cure rates. It seems reasonable to assume that the radiosensitivity of normal tissues should be regarded as a so-called complex trait depending on multiple clinical, life-style and genetic factors. However, studies have shown that clinical factors such as age, comorbidity, radiation dose and –volume can only partly explain the risk of late radiotoxicity [2]. In addition, abdominal surgery [85], diabetes mellitus [86] and acute toxicity [85] were identified as predictors for late side effects. Unfortunately consistent evidence in independent studies is lacking. Patients with late radiation toxicity are thought to have a constitutionally altered normal tissue response to ionizing radiation compared to patients without toxicity. Therefore, the hypothesis has been put forward that normal tissue radiosensitivity is mostly dependent on genetic factors [87].

Incidence of severe side effects
Patients may develop acute and/or late side effects, different in grade of severity. Acute side effects are defined as complications that occur during radiation treatment and disappear within 3 months after the start of therapy. Late side effects are complications that develop after 3 months or even years later after radiation treatment and are thought to be more severe and chronic. Among others, prostate cancer is very effectively treated by ionizing radiation (± hormonal blockade), leading to high survival rates. Prostate cancer is the second most common cancer in men worldwide [88], and the number of prostate cancer patients will further rise due to the increase in lifespan in Western societies. The introduction of widespread prostate-specific antigen (PSA) screening led to early detection of the disease, and resulted in an increased 5-year survival rate in many countries. The large number of patients with a relatively high survival rate enables the study and monitoring of late radiation toxicities. Approximately 10% of all treated prostate cancer patients will develop severe late complications (grade≥3) and approximately 10% moderate to severe complications (grade 2). Taken together, up to 20% of these patients experience moderate to severe side effects. The most severe toxicities caused by radiotherapy for prostate cancer are intestinal and rectal morbidities. Symptoms include rectal bleeding,
faecal incontinence, tenesmus, increased stool frequency, diarrhea and bowel/abdominal pain called the rectal syndrome.

Genetic factors in late toxicity

The basis for the differences in response to radiation is undoubtedly multi-factorial and validation of functional assays that integrate these responses is essential. Little is known about the role of genetic predisposition for late radiation damage in patients with cancer. The individual variability in normal tissue response after radiotherapy may be caused by subtle mutations in genes involved in the cellular response to ionizing radiation. The possible relationship between single nucleotide polymorphisms (SNPs) in candidate genes involved in apoptosis [89], DNA repair [90-94], steroid metabolism proteins [95] or fibrosis [96] and their role in the development of late radiation toxicity has been explored by several studies in a retrospective cohort of prostate cancer patients. SNPs in for example XRCC1, XRCC3 LIG4, and ERCC2 genes were identified as markers to predict individual risks for complications arising from radiotherapy. Unfortunately, an independent validation study of genetic variants and SNPs reported to be associated with radiation toxicity could not confirm any of the associations after multiple testing correction [97]. Differences in study set-up, size of patient cohort, ways of toxicity scoring (acute or late) and the correction for multiple testing might explain these conflicting results. The hypothesis that an individual SNP or mutation might serve as a clinical relevant marker seems is highly unlikely. Recently, a large GWAS study in over 700 prostate cancer patients identified one locus comprising TANC1 at 2q24.1 [98]. Two validation cohorts of 633 and 368 patients respectively also observed multiple SNPs located in this particular region pointed to an association between the TANC1 gene and the development of overall late radiation toxicity.

Apart from single nucleotide polymorphism, differences in gene expression have also been used to separate patients based on toxicity status. The genetic predisposition for late radiation toxicity of individual cancer patients can be investigated by assessing the radiation response in normal tissue cells with genome wide expression profiling (chapter 5 and 6). Svensson et al. [99] showed that the analysis of expression profiles using gene sets belonging to a functional network substantially improved the power of the analysis over the use of single genes. Based on the gene expression profiles of ex vivo irradiated lymphocytes, prostate cancer patients with and without severe radiation complication could be discriminated. Particularly gene sets involved in the apoptosis, ubiquitin and stress signaling networks were involved in the prediction of normal tissue damage after radiotherapy. Confirmation of this study in other patient cohort’s remains however difficult. The study of Finnon et al [100] showed that array analysis was sufficiently robust in detecting differential expression patterns in the radiation response of individual patients, but was unable to distinguish patients based on normal tissue complication status with
previously described gene sets. They do however suggest the use of gene expression profiles as clinically useful assays after further validation of specific signatures in prospective studies.

**Predictive test for normal tissue toxicity?**

Although results are conflicting and no reproducible or reliable predictive markers associated with late radiation toxicity have been identified yet [97, 101, 102], differences between patients in expression profiles, mutational status and *in vitro* radiosensitivity are detected. All these findings together indicate that there is indeed a genetic predisposition for late radiation toxicity and possible predictive factors associated with clinical responses to radiotherapy might exist. Genome wide screening together with functional studies, may augment the distinction between patient with and without late complication. Further research is warranted to uncover robust and validated predictive markers that can be used to assess the individual risk for late radiation toxicity before start of treatment.

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